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Primary CCR5 only using HIV-1 isolates does not accurately represent the in vivo replicating quasi-species

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Abstract

Most HIV-1 isolates depend on CCR5 or CXCR4 to infect target cells, and efficient use of other coreceptors is rare. We cloned HIV-1 envelopes from virus at acute infection and found that most use CCR3 efficiently. This result contradicts prevailing data, suggesting that CCR3 usage is rare. We hypothesized that direct isolation into PBMC biases selection of viruses that use CCR5 and not CCR3. We therefore compared coreceptor use of isolates obtained by PBMC coculture with envelopes cloned directly from patient blood samples, which should represent actively replicating species. Viruses derived by cloning generally used CCR3 and CCR5 with equally efficiently. In contrast, we found that viruses isolated by PBMC coculture largely, or exclusively, used CCR5. Regardless of whether CCR3 use contributes to HIV-1 transmission or pathogenesis, our results demonstrate that 'primary isolates' generated by PBMC culture are unlikely to accurately represent the in vivo replicating quasi-species.

Keywords: HIV-1; gp120; Coreceptors; CCR3; CCR5; Primary isolates

Introduction

CD4 and a chemokine coreceptor, usually CCR5 or CXCR4, mediate binding, membrane fusion and cellular uptake of human immunodeficiency virus (HIV)-1 and -2 (Clapham and McKnight, 2001). Viruses that use CCR5 (R5 phenotype) are present throughout the course of infection (Bjorndal et al., 1997; de Roda Husman et al., 1999; Fiore et al., 1994; Jansson et al., 1999; Long et al., 2002; McNearney et al., 1992). Viruses that additionally (R5X4 phenotype), or exclusively (X4 phenotype), use CXCR4 evolve during disease progression in about 50% of cases (Fiore et al., 1999; Karlsson et al., 2003; Scarlatti et al., 1997; Shankarappa et al., 1998). For HIV-1, other coreceptors such as CCR2b, CCR3, CCR8, CXCR6 and GPR15 have been shown to be used in vitro, albeit to much lower levels (Choe et al., 1996; Deng et al., 1997; Farzan et al., 1997; Rucker et al., 1997; Vodros et al., 2001). Broader and more efficient use of alternative coreceptors has been shown for HIV-2 (Clapham and McKnight, 2002; Deng et al., 1997; McKnight et al., 1998; Morner et al., 1999).

The relevance of infection mediated via 'minor' coreceptors is unclear, and it is generally thought that only CCR5 and CXCR4 play a significant role in vivo (Moore et al., 2004). However, CCR3 use has been implicated in microglial infection with a potential link to neuropathogenesis (Gabuzda et al., 1998; He et al., 1997). The prototype central nervous system (CNS)-derived viruses, HIV-1_{YU2} and HIV-1_{JRFL}, use CCR3 efficiently (Choe et al., 1996; He et al., 1997). However, we and others reported that CCR3 use may not be confined to brain derived viruses but may be a common feature in late or chronic infection (Dittmar et al., 1997; Ohagen et al., 2003; Peters et al., 2004).

Here, we show that CCR3 use, in addition to CCR5, is a common phenotype of in vivo replicating HIV-1 viruses. We also show that virus isolation by peripheral blood mononuclear cells (PBMC) coculture favors the growth of CCR5 only using strains, explaining why most "primary" HIV isolates do not use CCR3. The results suggest that current viruses and envelopes

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Table 1
Co-receptor use by early seroconverter Envs

Patient	Virus clone[a]	Isolation day[b]	Infectivity ($\times 10^4$ FFU/ml) for									
			NP2/CD4						U87/CD4			
			CD4 alone	CCR3	CCR5	CCR8	CXCR4	APJ	CD4 alone	CCR1	CCR2b	CCR5
MM1	1.2.1	28	0.03	67	62	0.5	0.1	–	–[c]	–	–	45
	1.2.3	28	–	51	87	–	0.04	0.01	–	–	–	69
	1.2.4	28	–	0.2	1.6	–	–	–	–	–	–	1.6
	1.5.58	84	–	19	130	0.8	0.05	0.02	–	–	0.01	150
MM2	2.5.2	32	–	350	210	10	0.1	–	–	–	–	48
MM3	3.2.2	18	–	0.1	0.5	–	–	–	–	–	–	0.3
	3.4.1	30	–	70	90	2.0	0.07	–	–	–	–	97
MM4	4.1.33	17	0.03	105	61	0.2	0.06	0.03	–	–	–	62
	4.1.34	17	0.02	0.6	0.4	–	0.02	0.01	nd[d]	nd	nd	nd
	4.4.48	52	–	117	93	0.3	0.02	0.02	–	–	–	97
MM8	8.2.50	12	0.03	120	140	11	0.03	–	–	–	–	71
	8.2.51	12	–	15	9	0.5	0.03	–	–	–	–	6
	8.4.51	32	0.02	1.0	27	0.5	0.1	0.01	–	–	–	23
MM23	23.2.B	15	nd	0.02	1.6	0.07	0.07	nd	nd	nd	nd	nd
	23.2.D	15	nd	2.1	9.3	0.03	0.03	nd	nd	nd	nd	nd
	23.2.E	15	nd	60	76	0.5	0.5	nd	nd	nd	nd	nd
	23.2.H	15	nd	16	30	0.02	0.02	nd	nd	nd	nd	nd
MM27	27.1.F	28	nd	3.5	100	0.2	0.2	nd	nd	nd	nd	nd
	27.1.G	28	nd	–	1.8	–	–	nd	nd	nd	nd	nd
	27.1.J	28	nd	4.0	180	0.3	0.3	nd	nd	nd	nd	nd
	27.1.K	28	nd	5.0	150	0.3	0.3	nd	nd	nd	nd	nd
MM28	28.1.5	6	nd	32	90	0.07	0.07	nd	nd	nd	nd	nd

[a] Full-length molecular clone containing gp120 cloned directly from uncultured patient PBMC, inserted into an HIV-1_{HXB2}-based vector.
[b] Time-point from which the patient envelope was amplified, in days from onset of symptoms characteristic of primary HIV-1 infection.
[c] –, ≤100 FFU/ml.
[d] nd, not determined.

(Envs) used in HIV vaccine research may not accurately reflect the most relevant HIV quasi-species.

Results

We first determined the frequency of CCR3 use by HIV-1 Envs present at seroconversion. The Env surface unit (gp120) was amplified from uncultured patient PBMC from eight seroconverting men who presented with symptoms of acute retroviral infection. The *env* sequences were inserted into an HIV-1_{HxB2}-based replication competent vector and sequenced to exclude any identical *env* clones. Sequence analysis classed the viral Envs as HIV-1 clade B (the *env* sequences have been submitted to GenBank, for accession numbers see Materials and methods). Virus was produced through 293T cells and coreceptor use determined on the NP2 and U87 indicator cell lines (Deng et al., 1996; Soda et al., 1999). Briefly, viruses were titrated on cells in triplicate, and the cells were stained for p24 expression 48 h later. All patient Envs were R5 tropic and none of them used CXCR4 efficiently (Table 1). The limited infection of CXCR4 expressing cells detected with some high titre viruses was more than 2-logs lower than the infection obtained on CCR5 expressing cells, and these Envs therefore are not classically dual tropic. More interestingly, 7/8 patients harbored viruses with Envs that in addition to CCR5 could use also CCR3 to a substantial degree, the exception being patient MM27.

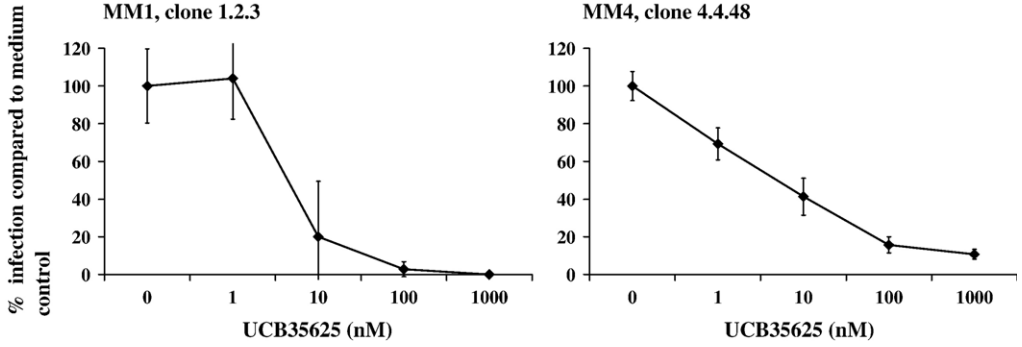

Fig. 1. The CCR3 antagonist UCB35625 inhibits infection of NP2/CD4/CCR3 cells. Infection of NP2/CD4/CCR3 cells by the Env-chimeric viruses can be blocked by increasing concentrations of the CCR3 antagonist UCB35625, verifying the specificity of the interaction. Error bars represent the standard deviation between triplicate wells.

Indeed, almost half of the Envs could use CCR3 as efficient as, or even more efficiently than, CCR5. For example, virus 1.2.1 from patient MM1 plated to 6.7×10^5 focus forming units (FFU)/ml and 6.2×10^5 FFU/ml on CCR3 and CCR5 expressing cells, respectively.

To verify that infection was mediated through an interaction with CCR3, we pre-incubated CCR3 expressing target cells with the CCR3 antagonist UCB35625 (1 h at 37 °C) (Sabroe et al., 2000). Almost 100% inhibition of the infection by viruses from MM1 and MM4 was achieved at higher (≥100 nM) concentrations of UCB35625 (Fig. 1). Infection of CCR5 expressing cells was unaffected at the same concentrations (data not shown).

HIV-1 envelopes from chronic infection also exhibit efficient use of the CCR3 coreceptor

Next we determined if the frequent and efficient CCR3 use is retained beyond seroconversion (at time-points between 316 and 833 days after onset of acute infection symptoms). To ensure that

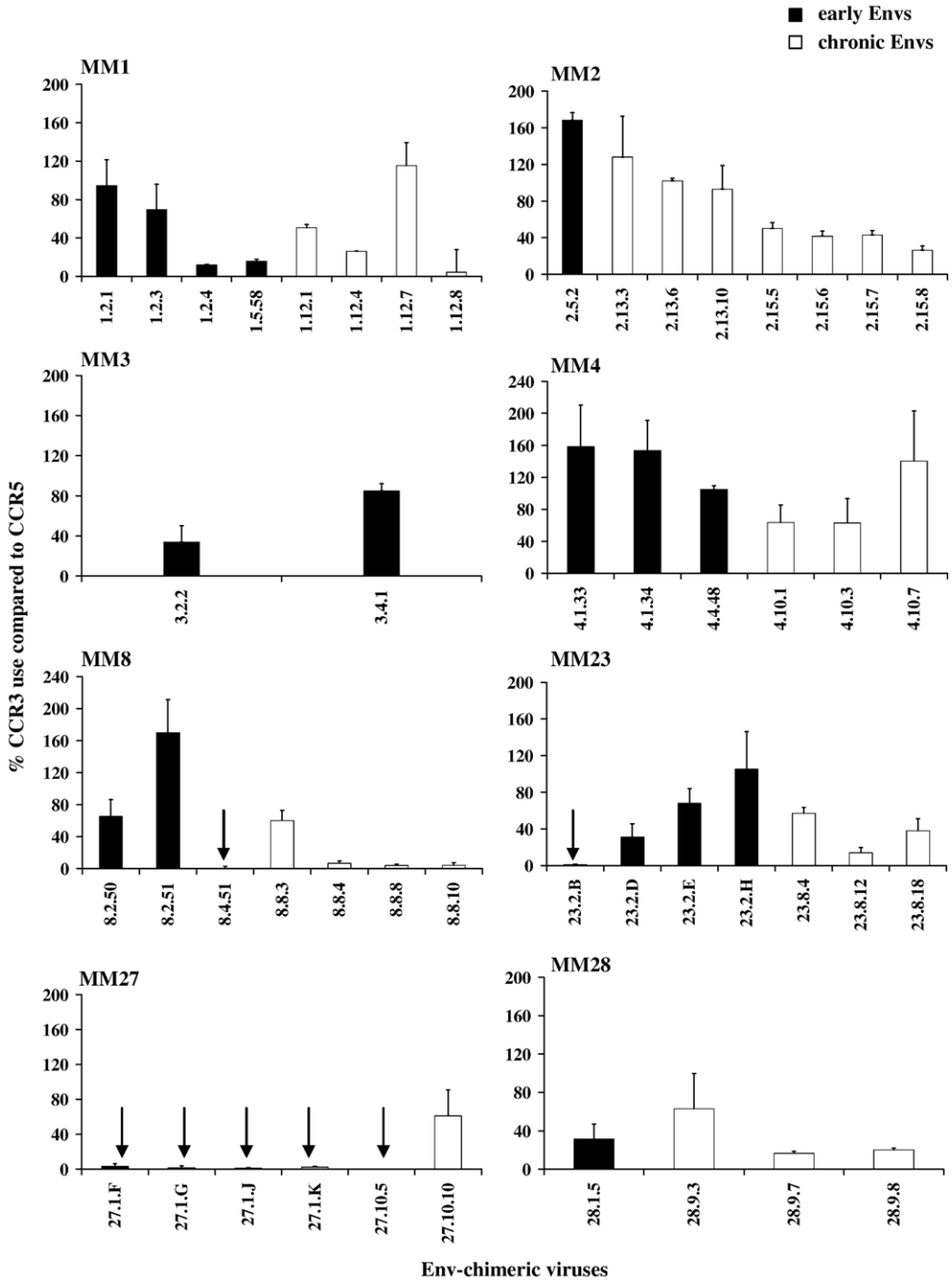

Fig. 2. CCR3 mediated infection by patient Env-chimeric viruses. The percentage of virus titer on NP2/CD4/CCR3 cells compared to NP2/CD4/CCR5 cells, of viruses with patient Env gp120 derived from early (black bars) and chronic (white bars) infection time-points. The bars represent the mean ratio for at least three independent experiments.

we sampled currently replicating virus, Envs from these chronic infection time-points were cloned from patient plasma viral RNA rather than PBMC as before. Fig. 2 shows that efficient CCR3 use was maintained in chronic infection in all patients. Each bar indicates the mean value for three or more independent experiments and error bars the standard deviation between experiments. Half (25 of 48) of all Envs characterized could use CCR3 within 2-fold of their CCR5 use. There was no significant difference in the level of CCR3 use, compared to CCR5 use, between Envs cloned from acute (median 33.8%) and chronic (median 50.0%) infection (Student's t test, $P > 0.05$). Interestingly, in MM27 where very limited CCR3 use was found in early infection, we saw gain of efficient CCR3 use with time.

Comparative experiments excludes assay artefacts

The efficient use of CCR3 throughout infection is contradictory to reported frequencies (Broder and Jones-Tower, 1999). However, the greater efficiency of CCR3 use described here is not due to a super-sensitive indicator cell. The efficiency of CCR3 use for HIV-1 viruses JRFL, SF162, YU2, SL2, 89.6, 2028, 2076, MN, NL4.3 and IIIB on NP2 cells was similar to those previously published (Fig. 3A) and clearly much less efficient than the majority of virus clones shown here (Fig. 2). The highest level of CCR3 (~30%) use was observed with HIV-1_{JRFL} and HIV-1_{YU2}, which previously has been described to use CCR3 'efficiently' (Choe et al., 1996; He et al., 1997). Furthermore, when we subcloned the Env surface unit (gp120) from a subgroup of the standard strains into our recombinant virus system, containing the trans-membrane unit (gp41) derived from HIV-1_{HXB2}, we observed no change in CCR3 use (Fig. 3B). Thus, we conclude that the efficient use of CCR3 that we observed with cloned Envs is not due to an artefact of our assay system.

Virus isolation through PBMC favors the growth of CCR5 only using viruses

Phytohemagglutinin and interleukin-2 stimulated PBMC are commonly used to isolate HIV in the laboratory. Only a small minority of these cells express CCR3 at their cell surface (unpublished data). In the above experiments, we show that direct amplification from either PBMC or plasma yields Envs that use CCR3. We reasoned that, in contrast to direct cloning of Envs, isolation methods using PBMC might select viruses using CCR5 only. To test this hypothesis, we isolated viruses by PBMC coculture from three patients (MM1, MM23 and MM27), at the same or similar time-points as the cloned Envs (Fig. 4). Despite MM1, MM23 and MM27 harboring "replicating" R3R5 viruses (left panel, Fig. 4), in the majority cases isolation into PBMC culture favored loss of CCR3 use for exclusive CCR5 use (right panel, Fig. 4). Apart for one isolate (MM23-1), none of the PBMC isolates could efficiently use CCR3 (range <1% to 6% of CCR5).

These results suggest that culture of HIV-1 through PBMC biases isolation of CCR5 only using viruses. To further strengthen this observation, we isolated 16 viruses from 11 more patients by PBMC coculture. The coreceptor usage of these isolates is presented in Table 2. Only one isolate, MM23-1, showed CCR3 use comparable to directly cloned Envs. The majority (10 of 16) displayed very limited CCR3 use (less than 10% of CCR5), supporting our hypothesis. Indeed, the difference in the level of CCR3 use compared to CCR5 use between cloned Envs (median 46.5%) and the isolates generated by PBMC culture (median 6.0%) was a highly significant (Student's t test, $P < 0.01$).

Discussion

Our results show that efficient CCR3 use by HIV-1 (clade B) is frequent in both early and chronic infection. Since PBMC isolation favors the outgrowth of isolates that use CCR5 exclusively, our results suggest that CCR3 use is positively maintained in vivo and selected against in PBMC culture. A possible positive selective force might be to colonize specific compartments. For example, microglial cells in the CNS express CCR3 that can mediate infection by some HIV-1 isolates (He et al., 1997). However, microglial

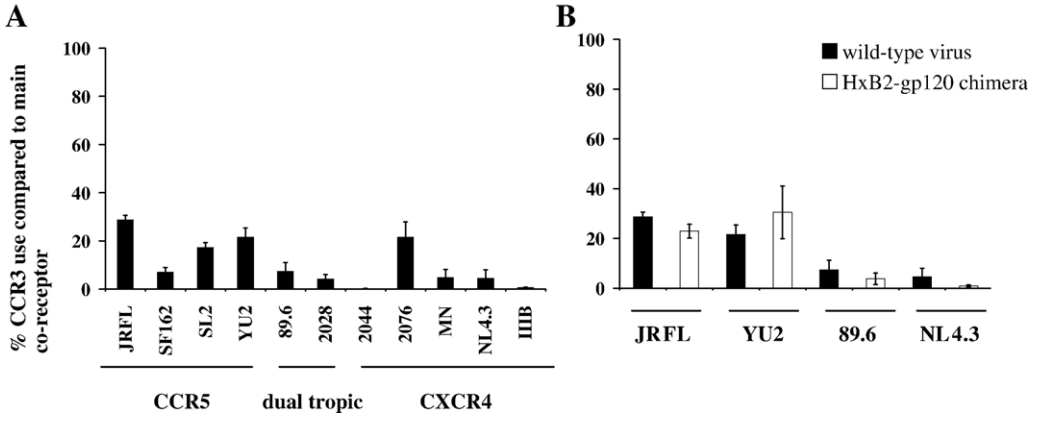

Fig. 3. Analysis of CCR3 usage by previously characterized HIV-1 viruses. (A) The mean percentage of CCR3 use compared to the main coreceptor use of viruses titrated on the NP2 indicator cell lines. The bars represent the mean ratio of at least two independent experiments and error bars the standard deviation between experiments. (B) Transfer of viruses' Env surface unit into the HIV-1_{HxB2} vector does not alter their ability to use CCR3 for infection of NP2 cells.

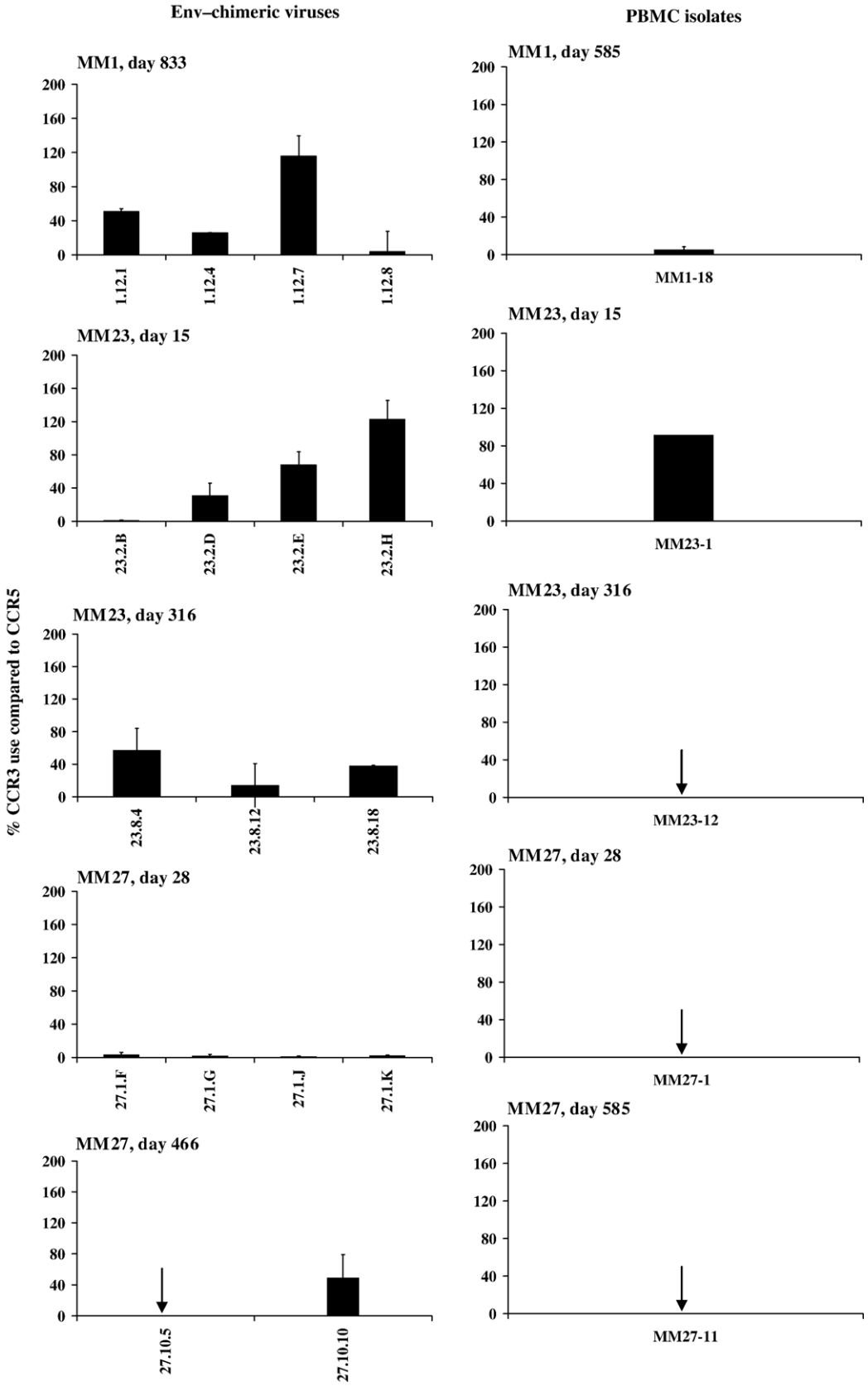

Fig. 4. Analysis of CCR3 usage by low passage primary isolates. Comparison of CCR3 use by patient Env-chimeric viruses and limited passaged patient PBMC isolates derived from similar time-points. In contrast to *env* cloning, virus isolation by PBMC coculture favors the growth of CCR5 only using viruses.

Table 2
Co-receptor use by limited passaged PMBC isolates

Patient	Virus isolate [a]	Isolation day [b]	Infectivity ($\times 10^3$ FFU/ml) for NP2/CD4			% CCR3 usage compared to CCR5
			CCR3	CCR5	CXCR4	
MM1	MM1–18	585	0.7	13	– [c]	5
MM14	MM14–1	40	–	17	–	<1
	MM14–9	519	–	90	–	<1
MM23	MM23–1	15	9.5	12	–	80
	MM23–12	316	1.7	195	–	<1
MM24	MM24–1	26	2.5	10	–	25
	MM24–10	464	0.2	1.1	–	14
MM25	MM25–2	31	0.5	630	–	<1
MM26	MM26–2	62	1.9	95	–	2
	MM26–11	384	0.4	6.7	–	6
MM27	MM27–1	28	3.1	120	–	3
	MM27–11	585	0.6	10	–	6
MM28	MM28–1	6	0.3	3.2	–	9
	MM28–10	503	0.1	0.6	–	16
MM33	MM33–1	26	0.8	4.0	–	20
MM34	MM34–2	25	0.5	3.0	–	17
	MM34–3	32	3.7	54	–	7
MM35	MM35–1	37	12	1100	22	1
MM36	MM36–1	6	0.1	12	–	<1
MM37	MM37–1	9	5.0	104	1.0	5
MM38	MM38–1	29	5.1	25	–	20

[a] Viruses obtained by co-culture of patient PBMC and healthy blood donor PBMC, after a maximum of 28 days of culture.
[b] Time-point from which the patient PBMC was derived, in days from onset of symptoms characteristic of primary HIV-1 infection.
[c] –, <100 FFU/ml.

tropism may not solely account for maintenance of CCR3 use in vivo; we and others have shown that CCR3 using Envs are also present in blood, lymph node, spleen and lung tissues (Dittmar et al., 1997; Martin-Garcia et al., 2006; Ohagen et al., 2003; Peters et al., 2004). Possible targets include eosinophils, macrophages and T helper 2 cells, which express CD4 and CCR3 either constitutively or during inflammation (Sallusto et al., 1998). Furthermore, CCR3 is up-regulated on blood monocytes in HIV-1 disease (Price et al., 2001).

CCR3 could play a role in the pathogenesis of HIV-1 infection especially in patients with tuberculosis, malaria or helminthic infections. In these diseases, T cell activation is skewed toward the T helper 2 type (Lawn, 2004; Santucci et al., 2004; Sarfo et al., 2004; Tkachuk et al., 2001), which express high levels of CCR3 (Sallusto et al., 1998) and may be more permissive to HIV (Maggi et al., 1994).

In summary, we have shown that the ability to efficiently use CCR3 is a common trait of the replicating HIV-1 quasi-species in vivo, and thus, CCR3 use could have a role in viral pathogenesis. However, regardless of the potential role of CCR3 use in vivo, our results show that isolation of HIV-1 through PBMC does not accurately reflect the virus population replicating in vivo. Indeed, the outgrowing 'primary isolates' may even be relatively rare in vivo. This finding could have implications on vaccine designs that focus on Envs that are dedicated to CCR5 use.

Materials and methods

Patient cohort

We analyzed the coreceptor usage of viruses from 18 HIV-1 infected men. Their likely route of exposure was sexual contact with other men. Blood samples were obtained both at seroconversion (6–84 days after onset of acute infection symptoms) and about 1 or 2 year after infection (316–833 days after onset of symptoms). Recent HIV-1 infection was diagnosed by the detection of HIV-1 genomes (PBMC proviral DNA or plasma RNA) in the presence, or absence, of an evolving antibody profile that subsequently became fully positive. All subjects declined antiretroviral therapy and remained treatment naïve throughout the study.

The study protocol was approved by the Camden and Islington NHS Trust Ethics Committee and written informed consent obtained from all subjects.

Virus isolation by PBMC coculture

Viruses were isolated by coculture of patient PBMC and phytohemagglutinin (PHA)-stimulated donor cells, following standard techniques. Briefly, PBMC were isolated from ethylenediaminetetraacetic acid (EDTA)-treated blood by density gradient centrifugation and donor cells were incubated with PHA (0.5 μg/ml) for 3 days prior to coculture. The cultures were maintained in interleukin-2 (20 U/ml; Roche, Lewes, UK) supplemented RPMI-1640 (Invitrogen, Paisley, UK) with 10% (v/v) Foetal Calf Serum (FCS; Invitrogen, Paisley, UK) and monitored for virus growth by p24 detection using the Vironostika HIV Uni-Form II Ag/Ab microelisa system (bioMérieux, Boxtel, The Netherlands). Fresh donor cells and medium were added every 7 days. Viruses were harvested after 7–28 days of culture.

Amplification of gp120 and generation of chimeric molecular clones

Viral envelopes from early infection time-points were amplified from proviral DNA from patient PBMC, as described previously (Aasa-Chapman et al., 2004). Briefly, the gp120 was amplified using the primers 988L+ 5′-GTAGCATTAGCGGCCGCAATAATAATAGCAATAG-3′, 943S+ 5′-CAA TAGYAGCATTAGTAGTAG-3′, 609RE− 5′-CCCATAGTGCTTCCGGCCGCTCCCAAG-3′ and 628L− 5′-TCATCTAGAGATTTATTACTCC-3′ for the first round. For the nested polymerase chain reaction (PCR), primers 626L+ 5′-GTGGGTCACCGTCTATTATGGG-3′ and 125Y− 5′-CAC-CACGCGTCTCTTTGCCTTGGTGGG-3′ were used, which contain BstEII and MluI sites (bold). The polymerase chain reaction (PCR) conditions were 30 cycles of 92 °C for 45 s, 45 °C for 45 s and 68 °C for 210 s, finishing with a 7-min elongation step at 68 °C. The same internal primers and PCR conditions were also used for the subcloning of *env* from previously characterized HIV-1 clones (HIV-1_{JRFL}, HIV-1_{YU2}, HIV-$1_{89.6}$ and HIV-$1_{NL4.3}$). Envelopes from chronic infection

time-points were amplified by nested reverse transcriptase PCR (RT-PCR) from viral RNA, extracted from plasma samples using the QIAamp Viral RNA Kit from Qiagen (Crawley, UK). The extracted RNA was first DNAse I treated (Promega, Southampton, UK) at 37 °C for 1 h and purified using the QIAquick PCR Purification kit (Qiagen, Crawley, UK). The same amplification protocol was used as for amplification from PBMC DNA, with the exception that the first amplification round was done using the Titan One Tube RT-PCR Kit (Roche, Welwyn Garden City, UK) and preceded by a reverse transcriptase step (50 °C for 30 min, 60 °C for 30 min).

The amplified env fragments were cloned into pGEM-T Easy or pCR2.1-TOPO (both from Promega, Southampton, UK) and subsequently transferred into pHxB2-MCS-Δ-env, by digestion with BstEII and MluI. Plasmid pHxB2-MCS-Δ-env allows incorporation of heterologous gp120 sequences from amino acid 38 (seven amino acids after the signal peptide) to six amino acids prior to the gp120/gp41 junction (McKeating et al., 1996). The resulting molecular clones encode replication competent viruses with gp41 derived from HIV-1_{HxB2}. Chimeric viruses were produced by transfection of 293T cells using Fugene-6 (Roche, Welwyn Garden City, UK).

Plasmid DNA sequencing

The *env* gene of infectious virus clones were sequenced using a cycle sequencing protocol using 3.2 pmol of primer and 500 ng of plasmid in accordance with the manufacturers instructions (Big Dye Terminator Kit 3.1; Applied Biosystems, Foster City, CA, USA). Both strands were sequenced using 5 primers each, to give an average 2- to 4-fold redundancy. The sequences and their chromatograms were assembled into a contig using Sequencher software to obtain consensus readings (Gene Codes Corp.; Ann Arbor, Mich., USA). The *env* sequences have been submitted to GenBank with accession numbers: AY29500.1–02.1, AY295206.1, AY295211.1, AY295214.1, AY295214.1, AY295219.1, AY295223.1, AY295225.1–26.1, AY295233.1, AY295235.1, AY295237.1, DQ425059-92.

Virus titration and coreceptor typing

Human glioma (NP2) and human mega-glioblastoma (U87) cell lines, stably transfected with CD4 and CCR1, CCR2b, CCR3, CCR5, CCR8, CXCR4 or APJ, were grown in Dulbelcco's modified Eagle medium (DMEM; Invitrogen, Paisley, UK) supplemented with 5% (v/v) FCS, 1 μg/ml puromycin and 100 μg/ml G418. The cells were seeded at 2×10^4 cells/well in 48-well plates, to yield semiconfluent layers the following day. Ten-fold serial dilutions of viral stocks were incubated in triplicate on the cells for 2 h at 37 °C. The cells were then washed once and cultured for 48 h. Infection was detected by p24 immunostaining, as detailed elsewhere (Aasa-Chapman et al., 2004). Briefly, fixed cells were incubated with mouse anti-HIV-1 p24 monoclonal antibodies (ADP 365 and 266, NIBSC, Potters Bar, UK; 1:40 dilution for 1 h) followed by a goat anti-mouse Ig antibody conjugated to β-galactosidase (Southern Biotechnology Associates; Birmingham, Alabama, USA; at 2.5 μg/ml for 1 h). After incubation with X-Gal substrate at 37 °C, infected cells appear blue and focus-forming units (FFU) are counted using light microscope. Each virus stock was titrated at least three times on each cell line. The level (percentage) of CCR3 use compared to the main coreceptor was calculated from average ratios obtained from three or more parallel titrations on CCR3 and CCR5 (or CXR4) expressing cells. Error bars indicates standard deviations between experiments. The statistical significance in differences in the level of CCR3 use between study groups was tested by Student's *t* test.

Blocking experiments

NP2/CD4/CCR3 cells and NP2/CD4/CCR5 cells were seeded as described above. One hour prior to infection with ~300 FFU of virus, the culture medium was removed and replaced with 100 μl/well of medium containing 0–1000 nM of the CCR3 antagonist UCB35625 (Sabroe et al., 2000). Each virus was plated in triplicate onto each dilution of UCB35625. Inoculates were removed after 2 h and infection detected 48 h later by p24 staining (as above).

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